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Breast cancer is a disease whose progression requires the involvement of many different cell types. These cell types, in addition to the mutated cancerous cells that initiate formation of the tumor mass, include non-cancerous blood vessel and connective tissue cells. These ancillary cell types, while not cancerous on their own, are required by the cancer cells in order for a tumor to grow beyond a very small size. Therefore, it is important to understand the interactions between the cancerous and non-cancerous cellular components of a breast tumor mass, since such interactions may serve as novel targets for therapeutic intervention.

In addition to studying the nature of the interactions between the cancerous and non-cancerous cells, it is essential to know the origin of the non-cancerous cells in a tumor mass. It has been assumed that these supportive cell types derive from regions of tissue adjacent to the site of carcinogenesis. This work examines a second possibility that is plausible on the basis of recent literature; namely, that a significant fraction of the non-cancerous cells in a tumor mass can derive from, in addition to adjacent tissue, the circulation of a tumor bearing animal.

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INTRODUCTION:

Breast cancer is a disease whose progression requires the involvement of many different cell types. These cell types, in addition to the mutated cancerous cells that initiate formation of the tumor mass, include non-cancerous blood vessel and connective tissue cells. These ancillary cell types, while not cancerous on their own, are required by the cancer cells in order for a tumor to grow beyond a very small size. Therefore, it is important to understand the interactions between the cancerous and non-cancerous cellular components of a breast tumor mass, since such interactions may serve as novel targets for therapeutic intervention.

In addition to studying the nature of the interactions between the cancerous and non-cancerous cells, it is essential to know the origin of the non-cancerous cells in a tumor mass. It has been assumed that these supportive cell types derive from regions of tissue adjacent to the site of carcinogenesis. This work examines a second possibility that is plausible on the basis of recent literature; namely, that a significant fraction of the non-cancerous cells in a tumor mass can derive from, in addition to adjacent tissue, the circulation of a tumor bearing animal.

BODY:

Specific Aim i. Determine if the presence of a tumor mass leads to the mobilization of endothelial precursor cells into the circulation.

Precedent for this idea can be found in the literature; for example, it has been demonstrated that myocardial infarction leads to an increase in the numbers of circulating endothelial precursor cells (EPCs), which subsequently aid in repair of the damaged heart [8]. In order to address this possibility directly, we isolated bone marrow from mice bearing single or bilateral subcutaneous MCF-7+Ras tumors, as well as from age- and sex-matched control mice injected subcutaneously with the Matrigel vehicle alone. After 21 days of tumor growth, we determined that the average tumor burden was 0.1±0.02 g and 0.2±0.06 g for mice bearing single and bilateral tumors, respectively. We analyzed the marrow cell populations using a number of assays that allowed us to identify alterations in the proportions of different cell types within the marrow.

By flow cytometric analysis, we observed a significant increase in Sca-1-positive cells in both the peripheral blood and bone marrow of mice bearing bilateral tumors when compared to control marrow derived from non-tumor-bearing animals (Figs. 2A and B). This observation is provocative, as the Sca-1 cell surface marker is associated with primitive marrow cells. Consequently, we further analyzed bone marrow cell populations by quantifying the percentage of Sca-1-positive cells that are also positive/negative for either CD31 (an endothelial marker), or Lin (a cocktail of hematopoietic lineage differentiation markers). We determined that a significant fraction of the Sca-1-positive population was comprised of CD31-positive cells in tumor-bearing mice. Sca-1 and CD31 double-positive cells represent endothelial precursor cells, thereby suggesting that the number of endothelial precursor cells is increased in the marrow of tumor-bearing mice. Additionally, we analyzed the Sca-1-positive bone marrow cell population using a cocktail of antibodies that recognize markers specific for differentiated hematopoietic lineages, including erythroid cells, monocytes, and granulocytes (Lin). We observed that a significant proportion of the increase in the Sca-1 population was attributable to the Lin-positive fraction, suggesting that a number of progenitors are committed to particular differentiation lineages.

We eliminated the possibility that macrophages were responsible for the observed increase in the Sca-1-positive population by staining for the CD11b antigen, which is specific for monocyte/macrophages. Staining for CD11b revealed no significant difference between the experimental and control marrow populations. In experiments that will be undertaken shortly, we shall determine the particular lineage(s) responsible for the increase in Sca-1-positive cell populations. The observed increase in Sca-1-positive bone marrow cells was reflected by corresponding changes in the peripheral blood of tumor-bearing mice (Fig 2B). Therefore, we would like to extend future analyses to include peripheral blood cells as well as bone marrow cells from tumor-bearing and control mice.

We also examined alterations in the hematopoietic fraction of the bone marrow by FACS analysis of the CD45 surface marker. We observed a statistically significant decrease in the percentage of CD45-positive bone marrow cells from mice bearing bilateral tumors when compared with marrow from control mice (Fig. 2A). This result raises the possibility that lineage commitment in the bone marrow of tumor-bearing mice is shunted from the hematopoietic compartment to a mesenchymal compartment. Since fibroblasts represent the dominant mesenchymal cell type in tumor-associated stroma, we sought to determine whether cells of this origin might also be detected in the bone marrow. To test the presence of fibroblast-like cells in the bone marrow, we determined the relative numbers of cells expressing collagen I. There was a modest increase in collagen I in tumor-bearing mice compared to Matrigel control animals; however, this increase was not statistically significant (Fig. 2B).

The flow cytometric experiments cited above provided a clear indication that the proportions of various stem/precursor cell populations in the bone marrow are shifted in tumor-bearing mice, ostensibly in response to signals released by the tumor. We have demonstrated,

using CD31 and Sca-1 double-labeling experiments, that levels of circulating endothelial precursors are increased in tumor-bearing mice, when compared to control mice. Future experiments will involve additional functional tests of the various subpopulations that exist in the bone marrow.

Specific Aim ii. Determine of bone marrow-derived circulating precursor cells can give rise to mesenchymal components of the tumor stroma.

A flurry of recent reports has made it clear that many adult tissues are populated by multipotent stem cells, and that these stem cells can arrive from distant sites via the circulation [1]. The prominent role played by stromal cell types within many human carcinomas has been demonstrated [2,3,4]. The origin of the stromal cells in these tumors has been unclear. While it has been assumed that the stromal cells are recruited from adjacent host tissue into the tumor, it is possible, in principle, that they may also originate from progenitor/stem cells that arrive in the tumor mass via the circulation.

I have speculated that a number of distinct stromal cell types may be recruited into the tumor mass from circulating progenitors. In initial studies, I demonstrated the presence of GFP (green fluorescent protein)-positive stromal cells in the tumor mass of recipient mice after intravenous injection of a large number of GFP-positive, unfractionated bone marrow cells derived from Rag-1-/- donor mice into recipient NOD/SCID tumor-bearing mice. Rag-1-/- donor cells were used here to minimize the likelihood of graft vs. host reactions. In this initial experiment, the NOD/SCID mice were not irradiated, and thus the endogenous marrow remained intact during the transplantation procedure. I believed that this initial experimental protocol, while successful in demonstrating the presence of GFP-positive cells in the tumor-associated stroma, grossly under-represented the extent to which circulating cells can give rise to tumor-associated stromal cells.

Given the observed paucity of GFP-positive cells in the tumor stroma, I was unable to efficiently estimate the relative extent to which the tumor mass recruited circulating cells when compared to other control organs in the transplanted mice. To address these difficulties, I reasoned that mice stably transplanted with GFP-positive marrow cells would better approximate the steady-state physiology of circulating marrow-derived cells present in a cancer-bearing patient and would therefore serve as more appropriate hosts in which to examine the extent of tumor-associated stroma derived from the circulation.

As a further refinement of the initial experimental protocol, I enriched the donor GFP-positive bone marrow for hematopoietic stem cells (HSCs) prior to injection into the recipient mice. I therefore stably transplanted GFP-positive bone marrow into NOD/SCID mice bearing small but palpable MCF-7+Ras tumors (<5mm in diameter) by injection of 5000 side-population cells (SP cells) into the recipient mice 4 hours after a single-dose 360 rads irradiation of these hosts. The SP bone marrow fraction is identified on the basis of preferential efflux of the Hoechst 3342 dye and is a commonly-used marker that enriches for the HSC fraction in murine bone marrow [5]. (Fig 1A)

Three weeks after the transplantation procedure, I sacrificed the mice and analyzed the tumor mass and other organs for the presence of GFP-positive cells. The presence of between 5 to 30% GFP-labeled cells in the peripheral blood of these mice confirmed that these mice had indeed been stably engrafted (Fig.1B). (We chose three weeks as our experimental end-point because this time is optimized for allowing sufficient engraftment of the labeled donor HSCs while still capturing the tumor in a log-phase of growth at a physiologically-acceptable size; the tumor was <7mm in diameter upon harvest.)

I observed a significant fraction of GFP-positive stromal cells in the tumor mass and little to no recruitment to control organs in the same mouse (Fig. 1C). Control organs examined include liver, brain, lung, spleen, skin, heart, and kidneys. I estimated that ~20% of the tumor stroma were circulation-derived cells, while <0.01% of the stromal cells in control organs were circulation-derived. One anticipated exception to this rule was the lung, where I observed significantly greater numbers of GFP-positive cells when compared to the other control organs.

I have previously demonstrated that this is an artifact due to physical lodging of the injected cells due to the tail-vein method of injection (data not shown).

This observation has two important implications. First, the lack of any significant recruitment of GFP-positive cells into control organs implies that the recruitment of GFP-positive cells is not simply a consequence of the systemic radiation that the mice were subjected to prior to the transplantation procedure. Second, the significantly-increased (by 3 orders of magnitude) presence of GFP-positive cells in the tumor-stroma, when compared to control organs, indicates that the recruitment is not due to a physical trapping of circulating cells within the tumor mass but is rather due specific chemo-attractive signals present within the tumor microenvironment.

I immunostained tumor sections with a pan-cytokeratin antibody to identify the MCF-7+Ras cancer cells. Importantly, the GFP-positive cells present in the tumor mass did not co-localize with the cytokeratin staining (Fig. 1C), thereby eliminating the possibility that fusion between the MCF-7+Ras cancer cells and circulating GFP-positive cells was responsible for the presence of GFP in the tumor stroma [6,7]. Ongoing experiments include extensive histological analysis by counter-staining GFP-positive cell types and will allow us to identify the specific cell type(s) recruited to these tumors.

Taken together, these additional results demonstrate i) perturbations of cell types within the bone marrow of tumor-bearing mice; specifically, an increase in the numbers of circulating endothelial precursor cells in tumor-bearing mice and ii) that marrow-derived stem cells are indeed recruited to the tumor stroma. These observations provide a solid foundation for future work as outlined in the proposal body.

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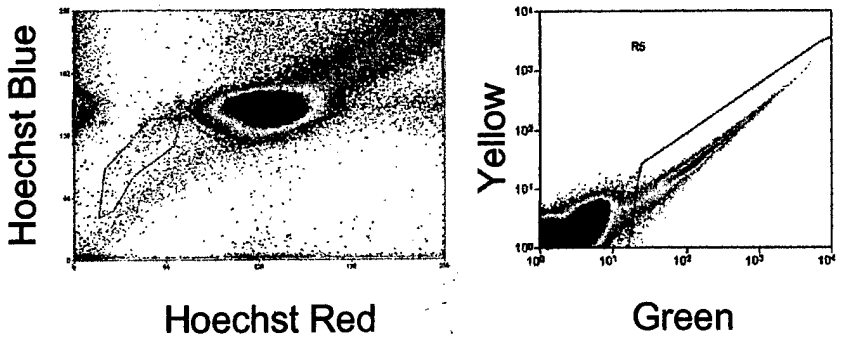
Certain of the experiments above were done in collaboration with two post-doctoral fellows in the laboratory-- Sandy McAllister and Anthony Karnoub.

Figure 1

A



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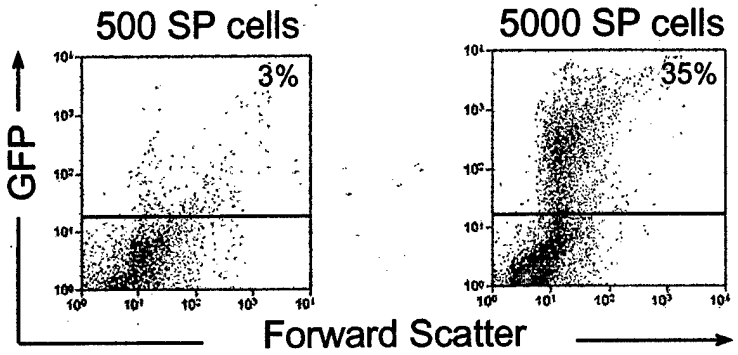

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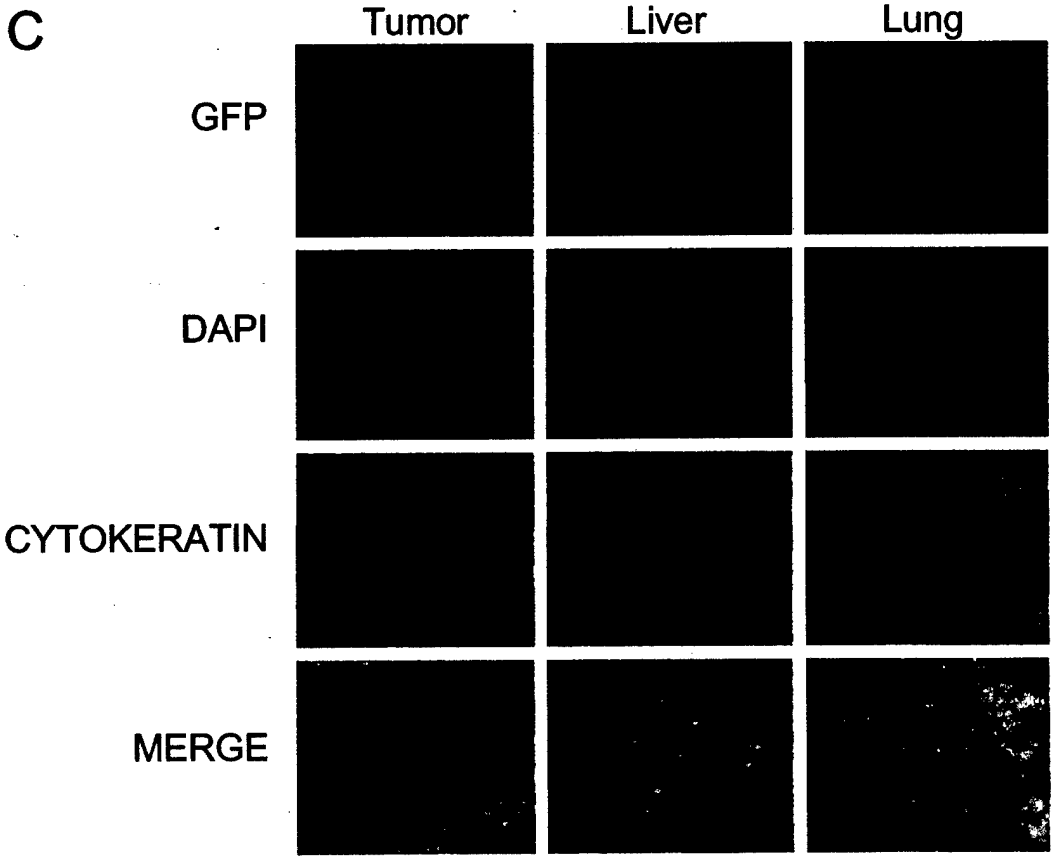

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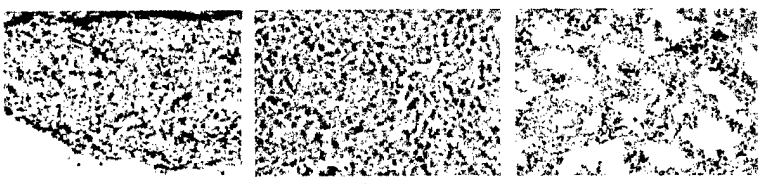
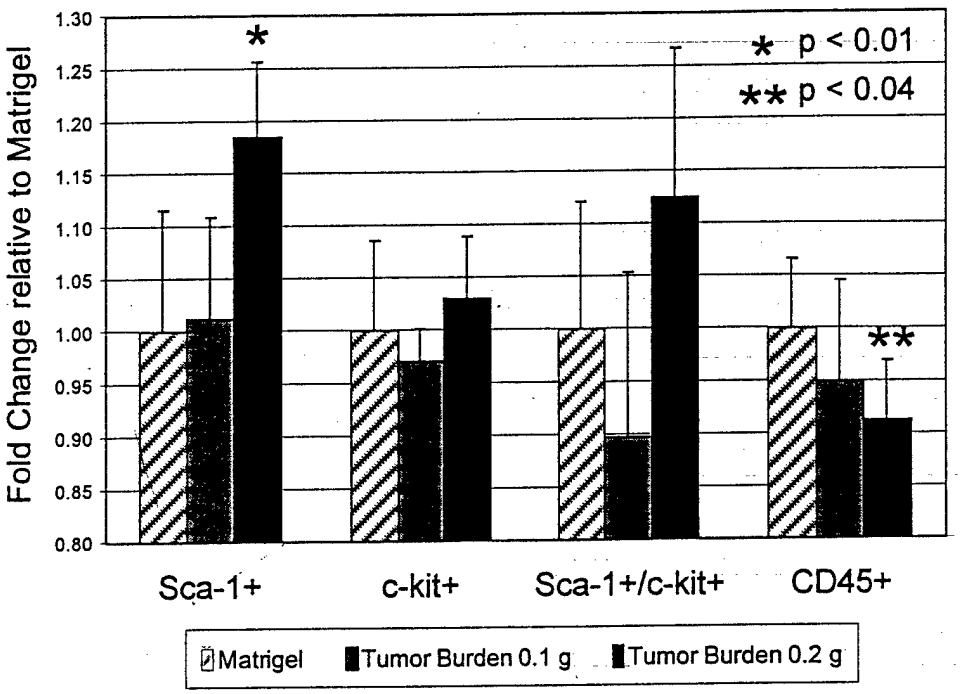


Supplementary Figure 1. Recruitment of Circulating Hematopoietic Stem Cells to a Tumor Mass. (**A**) *Isolation of SP cells from the bone marrow of a GFP-positive donor mouse and transplantation procedure.* Bone marrow cells freshly isolated from a donor GFP-expressing mouse, were sorted on a MoFlo Cell Sorter (Cytomation, Inc.) at the MIT Flow Cytometry Facility in the Center for Cancer Research. The HSC population was enriched by collecting side population (SP) cells based on their ability to efflux the vital dye Hoescht 33342. The left density plot indicates collection of SP cells by Hoechst 33342 emission at 450 nm (blue) and 670 nm (red). The right density plot represents collection of the SP cell population that is GFP-positive. Recipient mice were irradiated with 360 rads by a dual Cesium source 4 hours prior to injection of the SP cells. (**B**) *Engraftment of GFP-positive SP cells into NOD-SCID recipient mice.* 3 wk after intravenous or retro-orbital injection of either 500 or 5000 GFP+ SP donor cells into recipient NOD-SCID mice, peripheral blood was collected and subjected to FACS analysis to quantify the percentage of GFP+ cells in the circulation of recipient mice. (**C**) *Recruitment of GFP-positive hematopoietic stem cells to a tumor mass relative to other tissues.* 3 wk after delivery of 5000 GFP+ SP cells into recipient MCF-7+Ras tumor-bearing NOD-SCID mice, animals were sacrificed and all major organs and tumor tissue were harvested, fixed and sectioned. GFP+ cells (green) are visualized in tissues counter-stained with DAPI (blue) and pan-cytokeratin antibody (red) (20x magnification). The merged images demonstrate no overlap between the GFP-positive and cytokeratin-positive cells (**D**) Hematoxylin & Eosin staining of serial sections corresponding to the tissues in (C).

Figure 2

A



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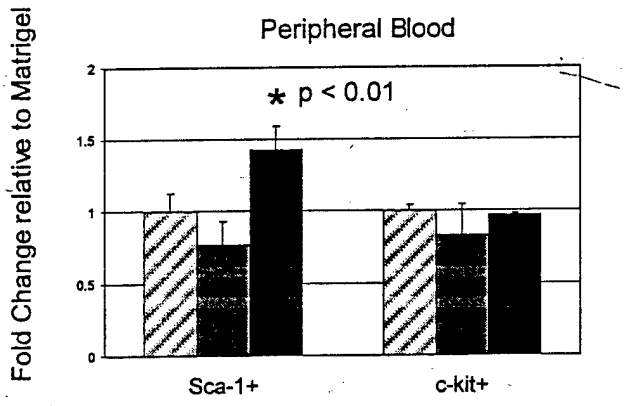

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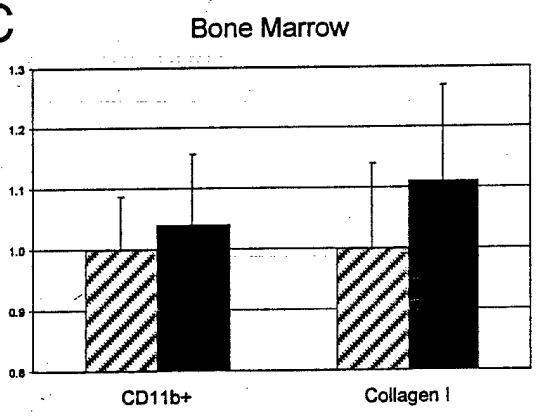

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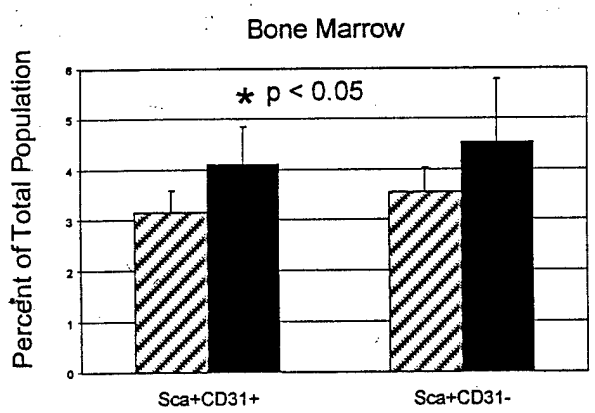

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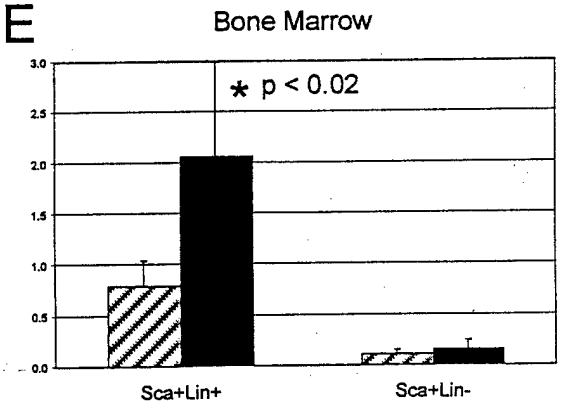

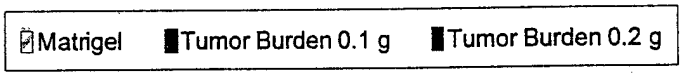

Supplementary Figure 2. Analysis of Bone Marrow from Tumor-Bearing Mice by Fluorescence Activated Cell Sorting (FACS). Bone marrow from control and tumor-bearing NOD-SCID mice was freshly harvested in PBS with insulin, dissociated using an 18 gauge needle, washed and resuspended in PBS for FACS analysis. The following anti-mouse antibodies were used for surface labeling of bone marrow cells: PE-Ly-6A/E (Sca-1), APC-CD117 (c-Kit), FITC-CD31, FITC-CD11b (PharMingen/BD Biosciences), APC-CD45 and PE-CD31 (Caltag Laboratories), collagen I (AbCam) with FITC-anti-rabbit IgG (Sigma), biotin-labeled Lineage Panel antibodies (CD45, CD3, Ly-6G, CD11, Ter-119; BD Biosciences) with FITC-streptavadin (get vendor). (**A**) *Flow cytometric characterization of BM-derived cells.* In all analyses in (A), the mean percentage of antibody-positive cells in the matrigel-injected control mice was normalized to 1. Mean percentage of tumor-bearing bone marrow cells expressing indicated surface marker is scaled accordingly and depicted relative to the matrigel-injected control. (**B**) *Representation of Sca-1+ and c-Kit+ cells in peripheral blood.* Peripheral blood was harvested from control and tumor-bearing mice and RBCs were lysed in lysis buffer (Sigma). Cells were washed and resuspended in PBS for FACS analysis. The alteration in the mean percentage of the Sca-1+ and c-Kit+ fraction (with respect to the entire population) in the tumor-bearing samples is shown relative to the matrigel-injected controls, normalized as described above. (**C**) *Representation of macrophage and fibrocyte precursor cell types in bone marrow.* Mean percentage of bone marrow cell populations expressing indicated surface markers from tumor-bearing mice is shown relative to matrigel-injected control mice, normalized as above. (**D, E**) *CD31 expression and Lineage-committed cells in the Sca-1+ population of bone marrow.* The bone marrow populations were further co-stained with the Sca-1 and CD31 markers, as well as the Sca-1 and Lin markers. Data are represented as mean percentage of the total bone marrow cell population.

KEY RESEARCH ACCOMPLISHMENTS:

1. Demonstration that there is, indeed, a perturbation of bone marrow-derived cellular subfractions in the marrow and peripheral blood of tumor-bearing animals.

2. Demonstration that there is an increase in the levels of endothelial precursors in the bone marrow and circulation of tumor-bearing animals.

3. Demonstration that a significant fraction of the tumor stroma is comprised of cells that are derived from the circulation of a tumor-bearing animal.

REPORTABLE OUTCOMES:

None to date.

CONCLUSIONS:

Work funded by this proposal has demonstrated an increase in the numbers of circulating precursor cells, including endothelial precursor cells in the marrow and circulation of tumor-bearing animals. This is significant, as it implies that specific signals deriving from the tumor mass lead to an increase in circulating levels of stem/precursor cells. It will be of interest to elucidate the precise nature of these signals. A future direction of the current work will investigate whether other cell types in addition to endothelial precursors are increased in the circulation in response to tumors.

The work funded by this proposal has also demonstrated that a significant number of circulation-derived cells are present in the stroma of breast cancers. This is of intrinsic interest, both from scientific and therapeutic point of view, and addresses a key question in breast cancer pathology. A further direction of the current work will be to precisely determine the nature of the recruited cells, as well as the precise contribution that these cells provide to tumor growth.

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